Azole resistance in *Aspergillus fumigatus*: a growing public health concern

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Purpose of review
Reports from the end of the 2000s forced the medical community to take azole resistance in *Aspergillus fumigatus* into account. Not only patients with chronic aspergillus disease, who develop resistance during long-term azole treatment, but also azole-naive patients are at risk, owing to the presence of resistant strains in the environment. The purpose of this review is to overview the latest findings concerning the origin, evolution, and implications of azole resistance in *A. fumigatus*.

Recent findings
TR_{34}/L98H is the predominant resistance mechanism of environmental origin in *A. fumigatus*. Recent epidemiological data show that this mechanism is an expanding problem, with reports from China, Iran, and India. However, the TR_{34}/L98H strains from the Middle East are genotypically different from the European isolates; their emergence is, therefore, not due to simple geographical spread of the 'European' isolates. A new environmental resistance mechanism, TR_{46}/Y121F/T289A, was detected in the Netherlands, conferring voriconazole resistance. In patients chronically treated with triazoles, the spectrum of resistance has become more diverse, with the emergence of non-*CYP51A*-mediated mechanisms. Central registration of treatment and outcome data of patients with resistant aspergillus disease are needed.

Summary
Azole resistance in *A. fumigatus* is evolving to a global health problem.

Keywords
aspergillosis, *Aspergillus fumigatus*, CYP51A, drug resistance, fungal

INTRODUCTION

Triazoles are the mainstay of therapy in infections with the opportunistic fungus *Aspergillus fumigatus*. The emergence of resistance is, therefore, of clinical concern. The first reports of patients with azole-resistant *A. fumigatus* isolates date from 1997, from patients receiving itraconazole therapy from Sweden [1] and California (isolates obtained in 1989) [2]. The characterization of two genes (*CYP51A* and *CYP51B*) encoding the azole target enzyme in *A. fumigatus*, sterol 14-α-demethylase, greatly contributed to the understanding of azole resistance mechanisms [3]. In the first decade after the discovery of azole resistance in *A. fumigatus*, only sporadic cases of resistance were published and resistance was considered an infrequent event. Two reports since the late 2000s changed this perception. First, in 2007, a series of Dutch patients – including azole-naive patients – were described with invasive aspergillosis due to pan-azole-resistant strains and resistance was attributable to one predominant resistance mechanism, TR_{34}/L98H [4]. This mechanism consists of a tandem repeat of 34 bases (TR_{34}) in the promotor of the *CYP51A* gene, leading to enhanced expression, combined with a leucine to histidine amino acid substitution (L98H) [4,5]. In 2009 a second report, from a specialized referral center for patients with chronic and allergic aspergillosis in Manchester, described resistance to have increased dramatically [6]. This situation differed from the TR_{34}/L98H-resistance problem in the Netherlands, as a variety of different *CYP51A*-related

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KEY POINTS

- Environmental resistance is a global and evolving public health problem, with TR_{34}/L98H described in India, China, and Iran and the emergence of a new resistance mechanism, TR_{46}/Y121F/T289A, in Europe.
- The spread of TR_{34}/L98H strains in Europe probably originated from a common ancestor, but they are genotypically distinct from isolates from the Middle East and India.
- Given an increasing number of reports suggesting a shift from *CYP51A*-mediated resistance to non-*CYP51A*-mediated resistance, research should be aimed at identifying new resistance mechanisms, such as the cdr1B efflux transporter, *HapE*, and *CYP51B*.
- The evidence for a fungicide-driven route of resistance development is increasing, but final proof is still lacking. Also the implication of withdrawal of certain fungicides for the dynamics of azole-resistant populations in the field is unknown.
- The revision of first-line therapy guidelines in regions with high (>10%) prevalence of resistance is still an open question; the central registration of the treatment and outcome of patients with resistant aspergillus disease is needed to guide this discussion.

resistance mechanisms were found [6]. These two reports reflect the two routes of resistance selection currently recognized. In patients with chronic aspergillus disease and long-term azole treatment, resistance develops 'in-patient' [6]. These patients were initially infected with a susceptible *A. fumigatus* strain, but this isolate evolved to a resistant phenotype under the selection pressure of azole treatment. The resistant isolate is isogenic to the initial infecting strain. Filamentous fungi are multicellular organisms, so a point mutation conferring azole resistance in one hyphal cell does not impact the phenotype immediately. However, if conidiospores (conidia) are produced from this mutated hyphal element, all conidia will harbor the mutation as will all hyphae that evolve from these spores [7]. A variety of resistance mechanisms (including *CYP51A* point mutations, especially in the hotspot regions at codon 54 or 220) in genetically unrelated strains have been described. In a follow-up study from Manchester, up to 17% of patients harbored a resistant isolate, with many isolates appearing to have a resistance mechanism that is not *CYP51A*-related [8]. This shift to non-*CYP51A*-mediated resistance mechanisms is not understood.

The epidemiology of azole resistance in the Netherlands suggested an alternative route of resistance selection. Evidence is accumulating that *A. fumigatus* becomes resistant in the environment. Patients are believed to inhale azole-resistant *A. fumigatus* spores and subsequently develop aspergillus disease that is refractory to medical triazoles. An important difference with the 'in-patient' route of resistance selection is the dominance of a single resistance mechanism, which has also been found in environmental isolates. In geographical areas with environmental azole resistance, azole-resistant invasive aspergillosis is observed. The risk of resistance selection in patients with invasive aspergillosis through azole therapy appears to be very low.

From 2007 through 2011, the TR_{34}/L98H resistance mechanism was also detected in six other European countries, including the United Kingdom [6], Spain [5], Belgium [9], Denmark [10], France [11], and Norway [12]. The geographical spread of TR_{34}/L98H is believed to be associated with the widespread use of agricultural fungicides [7]. As the selective pressure provided by these compounds in the environment continues, there is a need for strict monitoring of aspergillus resistance. The scope of this article is to overview the recent findings in this evolving field.

EPIDEMIOLOGY OF AZOLE RESISTANCE IN *ASPERGILLUS FUMIGATUS*

When comparing different studies reporting resistance epidemiology, the investigated subpopulation of patients should be taken into account, as higher resistance percentages are reported in patients under chronic azole treatment and different routes of resistance selection are involved. Another factor influencing comparability between resistance percentages is attributable to the difference between reported 'prevalence' (the percentage of patients with a resistant *A. fumigatus* among all patients with *A. fumigatus* cultured from a clinical isolate) or 'resistance rate' (the percentage of resistance among *A. fumigatus* isolates), the latter not corrected for duplicate sampling from the same patient. Table 1 gives an overview of the reported rates and prevalences of resistance.

In recent studies, the high prevalence of resistance in chronic and allergic aspergillosis reported in Manchester was also observed in other centers, with a prevalence of 4.5–8% in cystic fibrosis patients [10,18**]. In these patients, a variety of 'in-patient' resistance mechanisms were found, but also isolates harboring environmental resistance mechanisms. In France, resistance was seen more frequently in patients with previous triazole exposure, but the resistance mechanism involved was predominantly TR_{34}/L98H and the TR_{34}/L98H isolates were genotypically different from the initial infecting strain

Table 1. Rate of resistant isolates among clinical *Aspergillus fumigatus* isolates and prevalence of resistance in colonized or infected patients

Country, ref.	Study period	Study isolates	Resistance rate	Resistance prevalence	TR_{34}/L98H rate	TR_{34}/L98H prevalence
UK, [6]	1997–2007	Clinical isolates, irrespective of relevance; referral center for chronic/allergic aspergillosis	34/519 (6.6%)	20/400 (5%)	2/519 (0.4%)	2/400 (0.5%)
UK, [8]	2008–2009	Clinical isolates sent for susceptibility testing; referral center for chronic/allergic aspergillosis	64/230 (27.8%)	28/157 (17.8%)	0/230 (0%)	0/157 (0%)
The Netherlands, [12]	1994–2007	Clinical isolates, irrespective of relevance	63/2061 (3.1%)	45/1320 (3.4%)	–	39/1320 (3.0%)
The Netherlands, [13]	2007–2009	Clinical isolates, irrespective of relevance	82/1792 (4.6%)	63/1192 (5.3%)	74/1792 (4.1%)	57/1192 (4.8%)
The Netherlands, [14▪]	2009–2011	Clinical isolates, irrespective of relevance	–	63/921 (6.8%)	–	47/921[a] (5.1%)
Spain, [15]	2010–2011	Clinical isolates, irrespective of relevance	1/156 (0.6%)	–	–	–
Spain, [16▪]	1999–2011	Clinical isolates from proven or probable invasive aspergillosis or aspergilloma	6/343 (1.8%)	6/148 (4.1%)	0/343 (0%)	0/150 (0%)
Denmark, [10]	2007–2009	Clinical isolates from cystic fibrosis patients, irrespective of relevance	–	6/133 (4.5%)	–	2/133 (1.5%)
France, [17]	2006–2009	Clinical isolates from patients with hematological malignancy, irrespective of relevance	1/118 (0.8%)	1/89 (1.1%)	0/118 (0%)	0/89 (0%)
France, [11]	2010–2011	Clinical isolates from cystic fibrosis patients, irrespective of relevance	–	6/131 (4.6%)	–	2/131 (1.5%)
France, [18▪▪]	2010–2011	Clinical isolates from cystic fibrosis patients, irrespective of relevance	9/85 (10.6%)	4/50 (8.0%)	5/85 (5.9%)	3/50 (6%)
Germany, [19▪]	2011–2012	Clinical isolates irrespective of relevance	3.2% (17/527)	–	6/527 (1.1%)	–
Japan, [20▪]	1994–2010	Clinical isolates, irrespective of relevance (obtained from Pneumology Dept.)	11.2% (22/196)	–	0/196 (0%)	–
India, [21▪]	2005–2010	Clinical isolates from patients suspected of bronchopulmonary aspergillosis	2/103 (1.9%)	2/85 (2.4%)	2/103 (1.9%)	2/85 (2.4%)
Iran, [22▪]	2003–2009	Clinical isolates obtained from patients with aspergillus diseases	3.2% (4/124)	–	3/124 (2.4%)	–
USA, [23]	2001–2006	Isolates recovered from transplant recipients with proven or probable invasive aspergillosis	1/181 (0.6%)	–	–	–

Resistance rate: resistant isolates/all isolates tested; resistance prevalence: percentage of patients with a resistant strain among patients with *Aspergillus fumigatus* from a clinical sample (corrected for repeat sampling); TR_{34}/L98H rate: isolates with *CYP51A* mutation TR_{34}/L98H/all isolates tested; TR_{34}/L98H prevalence: percentage of patients with a resistant strain due to TR_{34}/L98H among patients with *A. fumigatus* from a clinical sample (corrected for repeat sampling).
[a]Thirteen of 921 resistant strains (1.4%) were attributable to the new environmental resistance mechanism TR_{46}/Y121F/T289A.

[18••]. If TR_{34}/L98H spores are present in the environment, patients with chronic (azole susceptible) aspergillus disease can still breathe in these spores during azole treatment and acquire 'environmental' resistance, before a phenotypically relevant mutation could develop 'in-patient'. The prevalence of an environmental resistance mechanism in patients chronically treated with azoles might be considered a pseudo-marker for the abundance of environmental resistance.

In 2012–2013, more countries reported TR_{34}/L98H, including the first reported cases from Germany [19•,24•,25], follow-up studies and cases from France [18••], the Netherlands [14•] and Spain [26•], and environmental presence of TR_{34}/L98H in Italy [27]. TR_{34}/L98H was also reported outside of Europe, in isolates from China, India, and Iran (Fig. 1), indicating that TR_{34}/L98H has become a global problem [21•,22•,28,29••,30]. As in-vitro susceptibility testing of clinical or environmental *Aspergillus* isolates is not routinely performed in many centers, azole resistance is probably still underestimated [22•].

The recovery of TR_{34}/L98H in many countries raises the issue of whether geographical (airborne) migration of resistant TR_{34}/L98H-bearing spores takes place, or there is independent local development and subsequent selection of TR_{34}/L98H in unrelated strains, or both. Recently, genetic markers were used to investigate 142 European isolates, which indicated that TR_{34}/L98H isolates showed less genetic variation than azole-susceptible isolates or those with a different genetic basis of resistance. This suggests a common ancestor of the TR_{34}/L98H mechanism that developed locally, possibly in the Netherlands, and subsequently migrated across Europe [31••]. In contrast, all Indian TR_{34}/L98H isolates (environmental and clinical) shared the same microsatellite genotype, indicating clonal spread, but this genotype was not recovered elsewhere [29••]. The authors hypothesized that the Indian genotype is an adaptive recombinant progeny derived from a cross between two strains, one from outside India, possibly azole-resistant, and a native strain from India, followed by rapid geographic migration [29••]. Clinical Iranian TR_{34}/L98H isolates genotypically also cluster separately from European isolates [22•]. A direct comparison of the Indian and Iranian isolates and more TR_{34}/L98H isolates from the Middle East would enhance our understanding of the origin and migration of TR_{34}/L98H [22•].

While TR_{34}/L98H is still spreading, a new environmental *CYP51A*-mediated resistance mechanism was described in the Netherlands, which migrated rapidly across Dutch hospitals [14•] and was also found in domestic homes. This new mechanism consists of a 46 bp tandem repeat together with substitutions (TR_{46}/Y121F/T289A) and is associated with voriconazole therapy failure. A fatal case of invasive aspergillosis due to a TR_{46}/Y121F/T289A strain was already described in neighboring country Belgium [32•]. Whereas TR_{34}/L98H leads to pan-azole resistance, with a pronounced loss of activity of itraconazole, TR_{46}/Y121F/T289A causes high-grade voriconazole resistance, with moderately attenuated (and variable) itraconazole and posaconazole minimum inhibitory concentrations

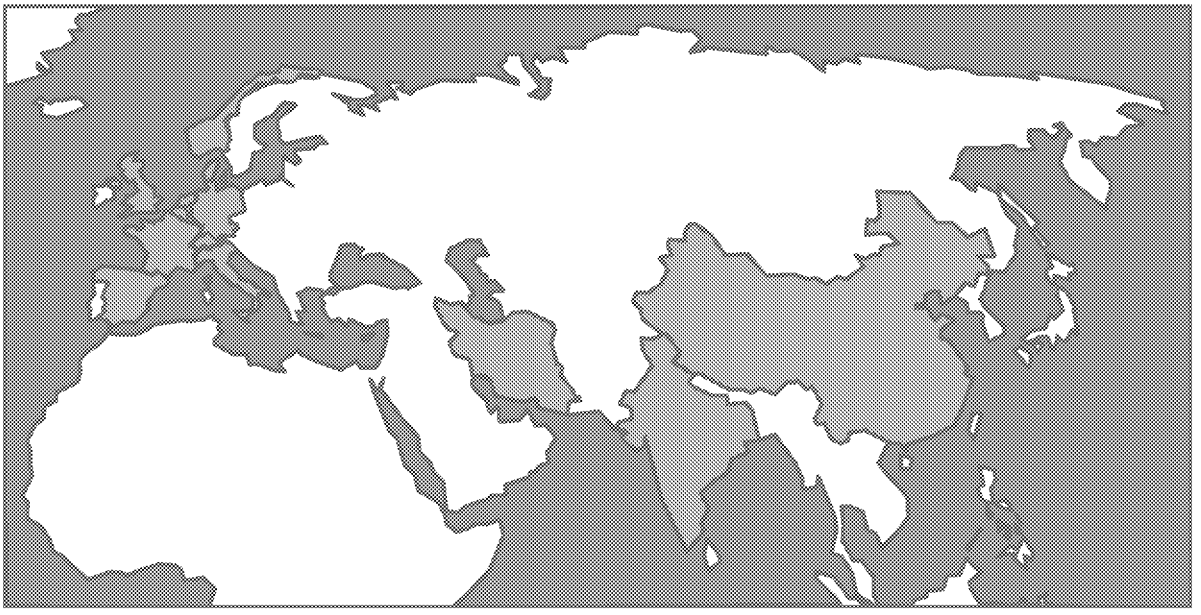

FIGURE 1. Geographical spread of the TR_{34}/L98H resistance mechanism (countries reporting TR_{34}/L98H marked in orange).

(MICs) [14▪]. Reduced therapeutic efficacy of itraconazole and posaconazole is certainly expected with TR_{46}/Y121F/T289A, but needs further documentation. The emergence of TR_{46}/Y121F/T289A underlines the continued selection pressure of azole fungicides in the environment. Surveillance studies are needed to monitor whether this new environmental resistance mechanism will further disseminate geographically.

NEW FUNDAMENTAL INSIGHTS INTO *ASPERGILLUS FUMIGATUS* RESISTANCE

The European Centre for Disease Prevention and Control (ECDC) very recently published a report integrating all evidence for a causal role of fungicides in resistance in *A. fumigatus* [33▪▪], which is an important step in the acknowledgement of this emerging problem not only within but also beyond the medical community. This is needed, owing to the potential implications of this issue for both human health and food security [34▪]. Table 2 summarizes the factors that support the hypothesis of a fungicide-driven route of resistance selection. As long as the molecular mechanisms at the origin of aspergillus' resistance are unclear, it will not be unraveled whether fungicides only provide selective pressure and aid in the spread of resistant strains, or really play a causal role in resistance development. The circumstances under which resistance selection might take place remain unknown. Genotyping studies indicate that the genetic diversity observed in TR_{34}/L98H is too large to be solely explained by asexual reproduction. Experiments demonstrated that TR_{34}/L98H strains can undergo a sexual cycle *in vitro* and cross with isolates of different genetic backgrounds [31▪▪]. Abundant phylogenetic incompatibility, consistent with recombination, was found in samples from India, supportive of sexual mating in natural populations of *A. fumigatus* [29▪▪]. However, it is unclear whether and in what conditions this phenomenon occurs in nature. It is, therefore, unknown whether this plays a role in the origin of the tandem repeat, as the underlying molecular mechanisms leading to the creation of tandem repeats are not well understood in general. The tandem repeat consisting of 46 bases in the recently emerging environmental voriconazole-resistant strains (TR_{46}/Y121F/T289A) includes the base sequence involved in TR_{34}, but with two additional flanking fragments. Tandem repeats are inherently unstable, but the mutation rate is also affected by external factors, such as the transcription rate and indirectly also by environmental stress [38]. There is no evidence that suggests that TR_{46}/Y121F/T289A evolved from a strain with TR_{34} to date. Microsatellite genotyping showed that TR_{46}/Y121F/T289A strains cluster together, but a different clade than TR_{34}/L98H and apart from wild-type control isolates [14▪].

In different studies, *A. fumigatus* with *CYP51A*-unrelated resistance was described, and the proportion of these isolates appears to have increased [8,19▪,20▪,28]. As a consequence, different genes are now investigated to identify the resistance mechanisms involved. Recent studies described a higher basal expression of the cdr1B efflux transporter [39▪], a mutation in the CCAAT-binding transcription factor complex subunit HapE [40▪▪] and high (both basal or azole-induced) CYP51B expression [41▪] to play a role in azole resistance. The relative importance of these different elements is yet unknown. There is also evidence that the *CYP51A* promotor has regulatory element(s) negatively influencing gene transcription, located upstream of the 34-base element involved in the tandem

Table 2. Evidence for a fungicide-driven route of resistance selection

	Reference
Agricultural triazole fungicides[a] have a comparable molecule structure to medical triazoles, binding to the same active site of the target enzyme	[35▪▪]
Bioinformatic studies suggest that the presence of L98H not only hinders the docking of the medical triazoles but also of the triazole fungicides[a]	[35▪▪]
TR_{34}/L98H also leads to resistance of agricultural triazole fungicides against *A. fumigatus*, in in-vitro susceptibility testing	[35▪▪]
The authorization of five triazole fungicides[a] for use in the Netherlands (1990–1996) preceded the first TR_{34}/L98H isolate (in 1998)	[35▪▪]
TR_{34}/L98H involves two genomic changes, which is unlikely to occur in a patient receiving azole therapy. The origin of tandem repeats is not well understood, but has also been found in phytopathogenic fungi, which lost susceptibility to azole fungicides.	[36▪]
TR_{34}/L98H isolates have been reported from geographical areas that correspond with the highest usage of azole fungicides (Arendrup)	[37]

[a]Propiconazole, tebuconazole, epoxiconazole, difenoconazole, and bromuconazole.

repeat [42**]. Full sequencing of the *CYP51A* promotor is, therefore, an interesting research option in aspergillus resistance of unknown origin.

IMPLICATIONS FOR PATIENTS WITH AZOLE-RESISTANT ASPERGILLOSIS

The increasing trend of resistance in several countries suggests the need for routine susceptibility testing of aspergilli from clinical isolates, in all centers caring for immunocompromised patients or patients with chronic or allergic aspergillosis. Early detection of resistance is important, especially in cases of invasive aspergillosis. In culture-positive patients, in-vitro susceptibility testing can take place; breakpoints have been published that enable interpretation of the MIC [43]. In culture-negative patients, detection of resistance remains problematic. Although resistance mutations have been detected directly in clinical specimens [44,45], the sensitivity of PCR is inadequate given the fact that the *CYP51A* gene is a single copy gene. Furthermore, the increasing diversity of CYP51A-mediated resistance mechanisms and the emergence of non-*CYP51A*-related resistance underscore the need for new (molecular) tools that enable the detection of a broad range of mechanisms.

A case series of patients with invasive aspergillosis caused by a TR_{34}/L98H isolate indicated a higher mortality rate compared with patients infected with a wild-type strain (88% versus 30–50%) [13]. Although some colleagues feel that revision of treatment guidelines is not yet indicated [46], in areas where resistance is widespread adjustment of the guidelines should be considered depending on the local epidemiology [47**]. Denning and Bowyer [47**] suggested that first-line therapy (with voriconazole) should remain unchanged, at least as long as the local resistance prevalence does not exceed 10%. In the setting of azole resistance, liposomal amphotericin B (L-AMB) is an important therapeutic alternative as no cross-resistance is described and L-AMB is recommended as an alternative first-line treatment in (azole susceptible) invasive aspergillosis (IDSA AI) and in salvage therapy (IDSA AII) [48]. Recent research examined the activity of different antifungal drugs and combinations against azole-resistant strains. The activity of AMB and caspofungin against resistant strains did not differ from the activity against wild-type strains in in-vitro experiments [49]. In a murine model of disseminated azole-resistant aspergillosis, L-AMB was equally effective against azole-susceptible and azole-resistant strains, independent of the underlying resistance mechanism [50*]. An alternative approach would be to start primary therapy with a combination of voriconazole and an echinocandin. Voriconazole and anidulafungin were synergistic in mice infected with voriconazole-susceptible *A. fumigatus*. However, in voriconazole-resistance (voriconazole MIC of 4 mg/l), only an additive interaction was observed [51*]. There is concern that in voriconazole-highly resistant *A. fumigatus* infection, the efficacy of the combination would rely only on that of anidulafungin [51*,52–54]. Anidulafungin monotherapy was only effective in 45% of mice infected with voriconazole-resistant strains, compared with 72% in mice infected with susceptible strains, raising questions about the value of this therapeutic alternative [53]. Anidulafungin is currently not approved for the treatment of invasive aspergillosis. At present, clinical data regarding alternative therapeutic options are very limited.

TOPICS FOR FUTURE RESEARCH

The growing problem of azole resistance in *A. fumigatus* should prompt research into the origin and management of resistance selection in the environment and on management strategies of patients with azole-resistant disease.

The origin and patterns of migration of TR_{34}/L98H and other environmental resistance mechanisms should be investigated by genotyping of resistant isolates from different areas of the world. This will help to understand the dynamics of migration. Field experiments are warranted that investigate the impact of withdrawal of certain fungicides on the population of resistant isolates within a wild-type population. The relation between azole fungicide exposure and the emergence of resistance mechanisms in *A. fumigatus* has not yet been proven.

For patient management, international surveillance networks are critical to determine the local epidemiology of azole resistance and to detect the emergence of new resistance mechanisms early. Diagnostic tools are urgently warranted that allow rapid detection of resistance mechanisms in culture-positive and, especially, in culture-negative patients. For this, it is important to identify all resistance mechanisms, including those not mediated through the *CYP51A*-gene.

Clinical data regarding the treatment and outcome of patients with resistant infection should be registered, to help in the re-evaluation of the current first-line management strategies in regions with a high resistance prevalence.

CONCLUSION

Resistance in *A. fumigatus* caused by environmental resistant strains is a growing public health concern

with global dimensions. A new environmental resistance mechanism was recently described and illustrates how the unchanged selection pressure of azole fungicides in the environment leads to an evolving situation. More research is needed on the geographical spread and molecular mechanisms of resistance development in *Aspergillus* and the exact role of fungicides herein. A network with central registration of resistance in *Aspergillus* and clinical data on alternative management strategies are warranted.

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Conflicts of interest

There are no conflicts of interest.

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